VERIFICATION OF TRANSLATION

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declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and

2. That the attached document is a true and correct translation made by me to the best of my knowledge and belief of:

(a) U.S. Provisional Patent Application No. 60/491,837
Entitled: "GENES THAT INCREASE REDIFFERENTIATION ABILITY OF PLANTS AND USES THEREOF"
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GENES THAT INCREASE REDIFFERENTIATION ABILITY OF PLANTS AND USES THEREOF

Technical Field

The present invention relates to the isolation and identification of genes that increase regenerative ability of plants, as well as methods for increasing regeneration ability, where these methods utilize these genes. The present invention allows improvement of the culture characteristics of plants, and development of transformation methods.

Background Art

Under appropriate conditions, differentiated plant tissue dedifferentiates to form calli (dedifferentiated cells) by undergoing cell division. Depending on the conditions, calli can redifferentiate to regenerate complete plants. The ability of such differentiated or dedifferentiated cells to regenerate individuals is called totipotency, and was first demonstrated in cultivation studies on tobacco and carrots in the mid 19th Century. Tissue culture techniques are based on this totipotency, and are widely used, especially in the field of plant breeding, to reduce the number of years to produce or breed new varieties by cell fusion and ovule culture, and to fix genetic traits and such. Recently tissue culture has become indispensable as an essential technology for basic research in artificial gene introduction (transformation methods). Transformation methods will certainly find use as even more powerful tools, as elucidating the function of individual genes by determining genomic nucleotide sequences attracts attention.

Totipotency is generally considered to be an ability retained by all plants, but in fact this ability is known to be easy or difficult to exercise, depending on the plant species, cultivar and organ. Compared to dicotyledonous plants, tissue culture and regeneration of monocotyledonous plants such as the major crops rice, barley, corn and so on is difficult, and studies of their cultures are greatly limited. In rice, relatively simple culture systems using ripe seeds have been established; however, varieties with sufficient regenerative ability are limited. In particular, palatable varieties Koshihikari, Sasanishiki, and IR line varieties widely cultivated in the tropics have low regenerative abilities, and regeneration of plants by tissue culture is problematic. If the regenerative ability of these varieties could be improved, not only could it be helpful in variety improvement and gene characterization studies, but the mechanism of the regenerative process might also be elucidated, and improvements in the regenerative ability of other unculturable plant species and varieties could also be expected.

Disclosure of the Invention

According to research to date, regenerative ability is a genetically controlled trait, and a number of quantitative trait loci (QTL) have already been reported. However, there are no reports of the successful isolation of regenerative ability genes from these gene loci. An

which contribute to plant regenerative ability. Another objective is to provide methods for improving plants by using the PSR1 genes.

First, prior to developing hybrid populations used to detect regenerative ability QTLs, the present inventors investigated the regenerative ability (callus weight and number of regenerated individuals) of each of a number of japonica and indica rice varieties. Of these, japonica rice "Koshihikari", with its extremely low regenerative ability, and indica rice "Kasalath", with its high regenerative ability, were selected (Fig. 1). F1 individuals were produced by crossing the japonica variety Koshihikari with the indica variety Kasalath, and these were backcrossed with Koshihikari as the recurrent parent, and then self-fertilized to produce the BC4F2 population. PCR markers were used to select from this population a total of nine lines comprising one or more domains of the short arm of chromosome 1, the long arm of chromosome 6, and the long arm of chromosome 9, which were already reported as Kasalath regenerative QTLs, and the regenerative ability of these lines was measured. Using map based cloning methods, regenerative genes were isolated from the calli DNA of an F3 population of about 5,500 individuals of the line showing the highest regenerative ability. The vast number of chromosomal regions was narrowed down to a 55kb PSR1 gene candidate region, and the genes in this region were analyzed. As a result, ferredoxin nitrite reductase, which is involved in nitrogen metabolism, was discovered. The nucleotide sequence of this ferredoxin nitrite reductase gene was determined in Kasalath and Koshihikari, these sequences were compared, and nucleotide sequence substitutions were discovered. The ferredoxin nitrite reductase gene encodes nitrite reductase, which has the action of converting nitrite into ammonia with ferredoxin as the electron donor. In previous physiological tests the cause of poor Koshihikari cultures was predicted to be calli impairment caused by accumulation of the toxic nitrite ion due to low nitrite reductase activity; however, these analyses identified the ferredoxin nitrite reductase gene as the gene causing poor Koshihikari cultures, and revealed that the regenerative ability of Koshihikari was improved by the action of the Kasalath ferredoxin nitrite reductase gene.

This allows efficient selection of traits indicative of high regenerative ability using molecular markers,, and can be applied to the cultivation and so on of highly regenerative varieties using molecular biology techniques and the like. Since the present genes are thought to be widely present in plants, they can be applied not only to rice, but to plants in general, and are thus thought to be of high industrial value.

Specifically, the present invention relates to the isolation and identification of genes that increase plant regenerative ability, and to plant improvements using these genes, and more specifically relates to:

[1] a DNA used to improve a regenerative ability of a plant, wherein the DNA is any one of (a) or (b)

(a) a DNA encoding a plant PSR1 gene

(b) a DNA that hybridizes under stringent conditions to a plant PSR1 gene, and that can

change a trait of a plant in the same way as (a);
[2] the DNA of [1] wherein the plant PSR1 gene is a rice gene;
[3] a vector comprising the DNA of [1] or [2];
[4] a transformed plant cell retaining the DNA of [1] or [2] in an expressible manner;
[5] the transformed cell of [4], wherein the plant is a rice;
[6] a transformed plant comprising the transformed plant cell of [4] or [5];
[7] a transformed plant that is a progeny or a clone of the transformed plant of [6];
[8] the transformed plant of [6] or [7], wherein the plant is a rice;
[9] a propagation material of the transformed plant of any of [6] to [8];
[10] a method for producing the transformed plant of any of [5] to [7], wherein the method comprises the steps of introducing the DNA of [1] or [2] into a plant cell, and then regenerating the plant from the plant cell;
[11] a method for improving the regenerative ability of a plant, comprising controlling an endogenous PSR1 activity in a cell of a plant;
[12] the method of [11], comprising introducing the DNA of [1] or [2] to the plant;
[13] the method of [11] or [12], wherein the plant is a rice.

The present invention showed that the regenerative ability of plants can be increased by regulating the activity of the *PSR1* gene product in plants. Therefore, this enables production of stable and highly regenerative varieties by regulating the activity of the *PSR1* gene product in plants.

The phrase "increase in regenerative ability" in the present invention means only that the ability of plants to regenerate under culturing conditions is increased, and the form of the regenerated individual is unchanged. This increase in regenerative ability allows the desired variety to be subjected to various cultivation experiments, and as a result, allows the efficient development of new varieties and functional analyses of genes.

In the present invention, the phrase "*PSR1* gene of plants" refers to a gene encoding ferredoxin nitrite reductase of plants. "*PSR1* gene of plants" comprises the rice *PSR1* gene (Fig. 4), and *PSR1* genes derived from other plants.

Methods for identifying an unknown "PSR1 gene of plants" can be carried out using hybridization techniques (Southern *et al.*, Journal of Molecular Biology 98: 503, 1975.); and polymerase chain reaction (PCR) techniques (Saiki *et al.*, Science 230: 1350-1354, 1985; Saiki *et al.*, Science 239: 487-491, 1988). That is, a person skilled in the art can isolate DNAs with high homology to the PSR1 gene from other desired plants and determine their sequences by using, for example, the nucleotide sequence of the rice PSR1 gene (Fig. 4) or parts thereof as a probe, and oligonucleotides hybridizing specifically to the nucleotide sequence of the PSR1 gene as a primer.

Hybridization reactions to isolate such DNAs are usually conducted under stringent conditions. Stringent hybridization conditions of the present invention include conditions such as 6 M urea, 0.4% SDS, and 0.5x SSC; and those conditions which yield similar stringencies. DNAs with higher homology are expected when hybridization is performed under conditions with higher stringency, for example, 6 M urea, 0.4% SDS, and 0.1x SSC.

Whether an isolated DNA encodes a PSR1 protein is usually determined from sequence homology. Sequence homology can be determined using the BLASTn (nucleotide level) and BLASTx (amino acid level) programs (Altschul *et al.* J. Mol. Biol.215:403-410, 1990). These programs are based on the BLAST algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990; Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). When using BLASTn to analyze nucleotide sequences, the parameters are score=100 and wordlength=12, for example. When using BLASTx to analyze amino acid sequences, the parameters are score=50 and wordlength=3, for example. Further, the Gapped BLAST program can be used as described by Altschul *et al.* when analyzing amino acid sequences (Nucleic. Acids. Res. 25:3389-3402, 1997). When using the BLAST and Gapped BLAST programs, the default parameters for each program are used. Specific techniques for these analysis methods are publicly known (http://www.ncbi.nlm.nih.gov.).

In the present invention the plants to be introduced with a trait of high regeneration by increasing PSR1 gene expression or PSR1 protein activity are not particularly limited so long as a desired plant whose regenerative ability is to be increased can be used; however, useful agricultural crops and ornamental plants are preferable. Examples of useful agricultural crops include monocotyledonous plants such as rice, corn, barley and wheat, and dicotyledonous plants such as rapeseed, soy, cotton, tomato and potato. Further, examples of ornamental plants include flowering plants such as chrysanthemums, roses, carnations and cyclamens.

When using the methods of the present invention to produce plants with high regenerative ability, a PSR1 gene encoding a highly active type of PSR1 protein is inserted to an appropriate vector, this vector is introduced to plant cells, and the obtained transformed plant cells are regenerated. Alternatively, plant cells are introduced with a construct incorporating a PSR1 gene encoding a highly active type of PSR1 protein under the control of an appropriate promoter which overexpresses in plants, and the transformed plant cells thus obtained are regenerated. Alternatively, they can be produced by producing introgression lines, and using molecular markers to select individuals introduced with a highly active type of PSR1 gene.

Those skilled in the art can introduce the DNAs of the present invention into plant cells using known methods, for example, Agrobacterium methods, electroporation methods, and particle gun methods.

In the case of the above-mentioned Agrobacterium methods, for example, the method of Nagel *et al.* can be used (Microbiol. Lett. 67: 325, 1990). According to this method, a recombinant vector is transformed into Agrobacterium bacteria, and then the transformed Agrobacteria are introduced to plant cells by known methods, such as leaf disc methods and the

like. The above-mentioned vectors comprise expression promoters such that, for example, the DNAs of the present invention are expressed in plants after their introduction to the plants. DNAs of the present invention are generally located downstream of these promoters, and terminators are then located downstream of the DNAs. Those skilled in the art can appropriately select recombinant vectors for use for this purpose, according to the method for introduction to the plant or the type of plant. The above-mentioned promoters include, for example, CaMV35S derived from cauliflower mosaic virus, corn ubiquitin promoter (Japanese Patent Application Kokai Publication No. (JP-A) H2-79983 (unexamined, published Japanese Patent Application)).

Further, examples of the above-mentioned terminators can include terminators derived from cauliflower mosaic virus, terminators derived from the nopaline synthase gene, and the like; however, so long as the promoters or terminators function in plants, they are not limited to these examples.

The plants introduced with a DNA of the present invention may be explants, cultured cells may be prepared from these plants, and the DNAs may also be introduced into the resulting cultured cells. The "plant cells" of the present invention include, for example, plant cells in leaves, roots, stalks, flowers, the blastodiscs of seeds and the like, calli, and suspension culture cells.

To efficiently select plant cells transformed by introduction of a DNA of the present invention, the above-mentioned recombinant vectors preferably comprise an appropriate selection marker gene, or are introduced to the plant cells along with a selection marker gene. Selection marker genes used for this purpose include, for example, the hygromycin phosphotransferase gene that gives resistance to the antibiotic hygromycin, neomycin phosphotransferase that gives resistance to kanamycin or gentamycin, and acetyl transferase gene that gives resistance to the herbicide phosphinothricin.

The plant cells introduced with the recombinant vectors are cultured in a known selection medium comprising a selection agent appropriate to the type of selection marker gene introduced. In this way, transformed plant cell cultures can be obtained.

Plants are then regenerated from the transformed cells introduced with the DNAs of the present invention. Plants can be regenerated from transformed plant cells by known methods, depending on the type of plant cell (Toki *et al.*, Plant Physiol. 100:1503-1507, 1995). For example, transformation and regeneration methods for rice plants include: (1) introducing genes into protoplasts using polyethylene glycol, and regenerating the plant body (suitable for indica rice varieties) (Datta *et al.*, in "Gene Transfer To Plants", Potrykus I and Spangenberg Eds., pp66-74, 1995); (2) introducing genes into protoplasts using electric pulse, and regenerating the plant body (suitable for japonica rice varieties)(Toki *et al.* Plant Physiol. 100: 1503-1507, 1992); (3) introducing genes directly into cells by particle bombardment, and regenerating the plant body (Christou *et al.* Bio/Technology, 9: 957-962, 1991); and (4) introducing genes using *Agrobacterium*, and regenerating the plant body (Hiei *et al.* Plant J. 6: 271-282, 1994). These

methods are already established in the art and are widely used in the technical field of the present invention. Such methods can be suitably used for the present invention.

Plants regenerated from transformed cells are then cultured in acclimation media. After that, the acclimated regenerated plants can be cultured under normal culture conditions to mature and bear seeds.

The presence of foreign DNAs introduced into transformed plants regenerated and raised in this way can be confirmed by known methods of PCR or Southern hybridization or the like, or by analyzing the nucleotide sequences of the DNAs in the plant.

In this case, DNAs can be extracted from transformed plants according to the known methods of J. Sambrook *et al.* (Molecular Cloning, 2nd Ed, Cold Spring Harbor Laboratory Press, 1989).

When using PCR or the like to analyze a foreign gene consisting of a DNA of the present invention which exists in a regenerated plant, an amplification reaction can be carried out using a DNA extracted from the regenerated plants as a template, as described above. Further, an amplification reaction can be carried out in reaction solution mixed with, as a primer, an oligonucleotide synthesized to comprise a nucleotide sequence appropriately selected based on the nucleotide sequence of a DNA of the present invention, or a DNA modified according to the present invention. In the amplification reaction, an amplification product which is a DNA fragment comprising a DNA sequence of the present invention can be obtained by repeating DNA denaturing, annealing, and extending reactions. Each type of amplified DNA fragment can be fractionated from reaction fluid comprising the amplification products by using agarose electrophoresis, and those DNA fragments can be confirmed to correspond to the DNAs of the present invention.

Having obtained a transformed plant containing a DNA of the present invention in its chromosome, it is possible to obtain a progeny of the plant by sexual or asexual reproduction. It is also possible to obtain reproductive material (such as seeds, fruits, spikes, tubers, tuberous roots, stubs, calli, and protoplasts) from the plant or a progeny or clone thereof, to mass-produce the plant based on such material. Thus, the present invention includes plant cells to which the DNA of the present invention has been introduced, plants containing these cells, progenies and clones of these plants, as well as reproductive material of the plants and their progenies and clones.

The present invention provides DNAs encoding rice PSR1 proteins. These DNAs may be useful for promoting the growth of cultured plant cells. Transformed plants can be produced using a DNA that encodes a rice PSR1 protein according to the above-mentioned methods. Specifically, the DNA is inserted into an above-mentioned vector, the vector is introduced to plant cells, and plants can be regenerated from those plant cells. Further, molecular markers can be designed based on a sequence of a DNA that encodes a rice PSR1 protein of the present invention, and plants comprising the DNA can be selected from a crossed population by using these molecular markers.

In the present invention, a "rice PSR1 protein" comprises not only proteins encoded by the DNA shown in Fig. 4, but also rice-derived proteins that are functionally equivalent to this protein. Examples of this kind of protein include artificially produced proteins, endogenous rice proteins, and so on. Herein, "functionally equivalent" means the target protein has nitrite reductase activity, or an activity of elevating the regenerative ability when introduced to a plant. These kinds of proteins comprise, for example, mutants, homologs and variants of the protein encoded by the DNA shown in Fig. 4.

Proteins functionally equivalent to a rice PSR1 protein can be produced using, for example, methods known to those skilled in the art for introducing mutations to an amino acid sequence in a protein (for example, site-directed mutagenesis (Ausubel *et. al.*, Current Protocols in Molecular Biology edit. Publish. John Wily & Sons, Section 8: 1-8.5, 1987)). Further, endogenous rice proteins produced by natural amino acid mutations can be isolated using hybridization techniques, polymerase chain reaction (PCR), and the like, based on the sequence information shown in Fig. 4.

The present invention comprises proteins with one or more amino acid substitutions, deletions, insertions and/or additions to that amino acid sequence (the amino acid sequence encoded by the DNA shown in Fig. 4), so long as the protein has a function equivalent to that of a rice PSR1 protein. There is no limit as to the number or site of mutations in the protein, so long as a protein function is maintained. In terms of preserving protein function, substituted amino acids are preferably amino acids comprising characteristics similar to the amino acid prior to substitution. For example, since Ala, Val, Leu, Ile, Pro, Met, Phe and Trp are characterized as non-polar amino acids, they are thought to comprise similar characteristics. Examples of uncharged amino acids include Gly, Ser, Thr, Cys, Tyr, Asn and Gln. Examples of acidic amino acids are Asp and Glu, and examples of basic amino acids are Lys, Arg and His.

Further, in the present invention, the DNAs encoding rice PSR1 proteins are not limited in their forms so long as they can encode an above-mentioned protein, and include cDNAs, genomic DNAs, chemically synthesized DNAs, and so on. Further, the DNAs include those with an arbitrary nucleotide sequence based on the degeneracy of the genetic code, so long as they encode a rice PSR1 protein. The DNAs encoding the rice PSR1 proteins of the present invention can be isolated by routine methods, such as hybridization methods using the DNA sequence shown in Fig. 4 or a part thereof as a probe, or by polymerase chain reaction (PCR) using primers designed based on information about these DNA sequences. Those skilled in the art can easily use known technologies to prepare these probes and primers based on DNAs encoding the rice PSR1 proteins of the present invention.

Brief Description of the Drawings

Fig. 1 is a photograph showing cultures of mature seeds of Kasalath and Koshihikari.

Fig. 2 shows the results of testing regeneration in two BC4F2 strains (C and D) showing strong regenerative ability.

Fig. 3 is a diagram showing a high density linkage map and physical map of a PSR1 gene candidate region.

Fig. 4 shows a genomic sequence of a PSR1 gene.

Best Mode for Carrying Out the Invention

Herein below, the present invention will be specifically described using examples, however, it is not to be construed as being limited thereto.

In the present examples, Kasalath and Koshihikari are tested as the present experimental materials. In testing regeneration, the number of individuals regenerated per gram of callus was compared after each individual was subjected to callus-inducing culture for one month and then to regeneration-inducing culture for one month. Further, in linkage analysis and creating physical maps, japonica rice "Koshihikari" was crossed with indica variety "Kasalath", backcrossed using "Koshihikari" as the recurrent parent, and then self-fertilized to cultivate a BC4F2 population, of which 5,500 individuals were tested. Using molecular markers closely linked to PSR1, clones comprising the gene were selected from a "Kasalath" BAC library.

[Example 1] Identifying the PSR1 gene

Japonica rice "Koshihikari" is a difficult variety to culture since calli turn brown during the culture process and growth is reduced. On the other hand, plants of the indica rice "Kasalath" have high regenerative ability and show vigorous calli growth. Thus, by crossing with Kasalath, Koshihikari is granted high regenerative ability. To identify the gene causing this, nine lines of plants with the Koshihikari genomic background and with fragments of the Kasalath genome inserted into one or more domains on the short arm of chromosome 1, the long arm of chromosome 6, and the long arm of chromosome 9, which were already known as the Kasalath regenerative ability QTL, were selected from the BC4F2 population using PCR markers. Regeneration tests were then carried out on these selected plants. As a result, only two lines showed vigorous regenerative ability, and both comprised the Kasalath genome in the short arm region of chromosome 1. From the above results, the PSR1 gene, which governs regenerative ability in rice, was determined to exist on the short arm region of chromosome 1.

To clarify the structure and function of the PSR1 gene in terms of molecular biology, map based cloning was used to isolate PSR1 genes. Using one BC4F2 line showing the greatest regenerative ability, 100 F2 plants were developed and their DNAs were extracted, then molecular markers were used to carry out linkage analysis, and the PSR1 genes were thus revealed to be situated at about 72 cM on the short arm of chromosome 1. Further, to determine the position of the PSR1 gene in more detail, 5,500 F2 plants were used to carry out high-resolution linkage analysis, and as a result the PSR1 gene was revealed to be within a 55kb region between P152 and P195. To select genomic clones comprising PSR1 gene, a Kasalath BAC library was screened, and BAC clones comprising a PSR1 gene were selected (Fig. 3). Gene prediction was carried out on this 55kb, and the ferredoxin nitrite reductase gene, thought

to be involved with the culture characterstics of rice, was discovered (Fig. 3). Thus, the nucleotide sequences of the Kasalath and Koshihikari ferredoxin nitrite reductase genes were determined, and nucleotide substitutions were discovered (Figs 3 and 4). From the above results, the PSR1 gene was concluded to encode the ferredoxin nitrite reductase gene.

Industrial Applicability

Recently, studies utilizing transformation methods for the development of useful plants and for functional analyses of genes are progressing rapidly. Since transformation methods allow the use of genes beyond the confines of biological species, which is impossible in conventional breeding based on crossing and selection, novel plants may be produced. Furthermore, as genomic sequences are elucidated one after another, transformation methods are also being used for gene disruption, expression regulation analysis, and such to elucidate the function of each gene. Generally, when producing a plant transformant, a plasmid vector comprising both the gene to be introduced and a drug resistance marker gene such as an antibiotic resistance gene is introduced into plant cells by the Agrobacterium method or by electroporation, and transformed cells are selected by drug treatment. The transformed cells that are selected regenerate into plant bodies through cell growth. Thus, to utilize such transformation methods, tissue culturing techniques must be established. Tissue culturing techniques are extremely useful not only in transformation methods, but also in mutant production using somaclonal variation, cultivar breeding using cell fusion or ovule culture, fixation of hereditary character, shortening of the number of years taken for breeding, and the like.

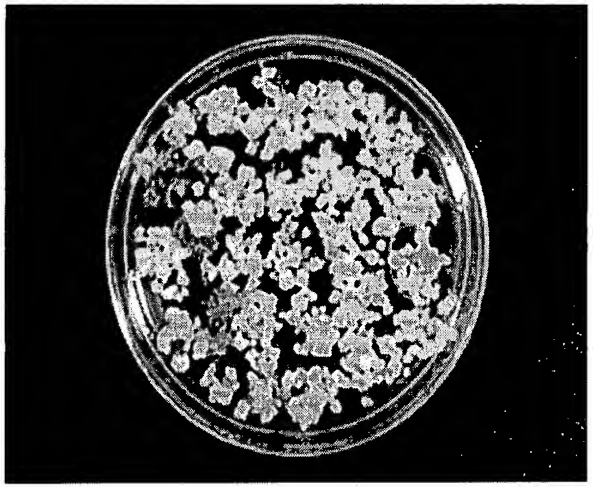
The major grain for which culturing techniques are most utilized is rice, but the presence of large differences in culturing characteristics between varieties is considered a problem. In particular, it is difficult to culture the major varieties in Japan, such as Koshihikari and Akitakomachi, as well as many indica varieties cultivated in the tropics, and therefore these varieties cannot be directly used as materials for tissue cultures. These differences in culturing characteristics between varieties are phenomena commonly observed in a number of plants and is not limited to rice, but there has been no progress in elucidating the phenomena.

The present inventors isolated genes involved in regeneration ability, enabling efficient selection of highly regenerative traits by using molecular markers (marker selected breeding), and enabling improvement of regeneration ability using molecular biological methods (molecular breeding). Furthermore, when introducing a gene into varieties that are difficult to culture, a *PSR1* gene can be used as a selection marker; and the acquired regeneration ability can be used as a marker trait.

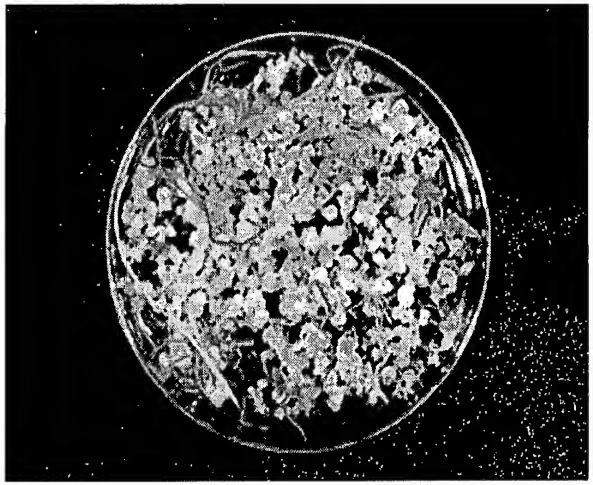
Grains such as rice, corn, wheat, and barley are major energy sources for humans, and

are the most important plants for humans. These grains all belong to the family *Poaceae*, and seem to have evolved from a common ancestor. They have high genetic homology (genomic synteny) with one another. Of these grains, rice has the smallest genome, and this is why rice is used as a model plant for grains. Rice genes are present in the genomes of rice relatives such as wheat and corn, and genes isolated from rice can be easily isolated from wheat and corn. In addition, rice genes can be applied directly to grain breeding of wheat, corn, and such. Therefore, the present genes may be applied not only to rice but also to wide varieties of plants.

KOSHIHIKARI



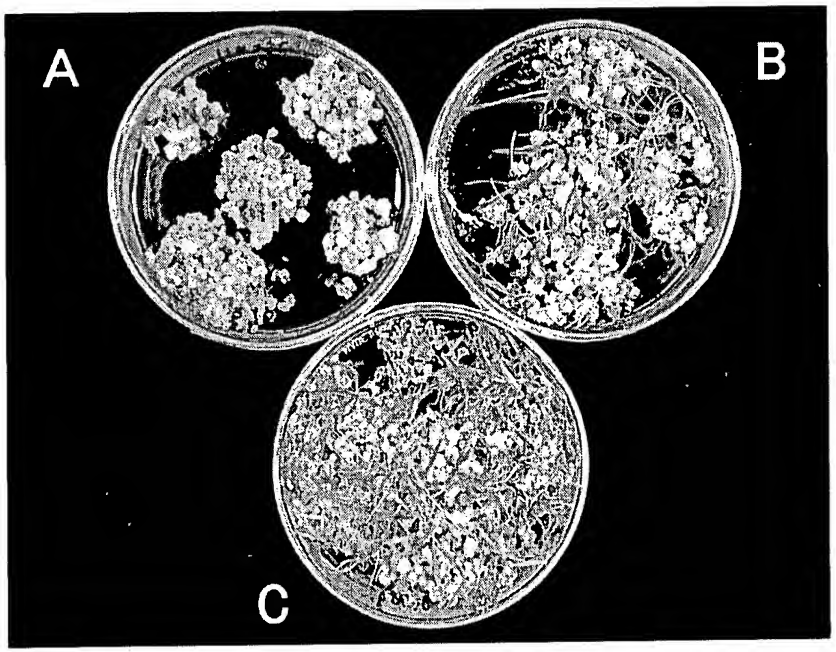
KASALATH



SEEDS	NUMBER OF GRAINS	WEIGHT OF CALLUS AT COMMENCEMENT OF REGENERATION (g)	WEIGHT OF CALLUS AT TIME OF TEST (g)	NUMBER OF SHOOTS	NUMBER OF SHOOTS/GRAIN	NUMBER OF SHOOTS / WEIGHT OF CALLUS AT TIME OF TEST
KOSHIHIKARI	32	0.92	14.74	11	0.34	0.75
KASALATH	32	1.31	32.24	505	15.78	15.66

FIG. 1 Regeneration test for Koshihikari and Kasalath

Mature seeds were cultured for one month in calli-inducing medium and
the calli produced were transferred to regeneration medium where culture was continued for one month.



SEEDS	NUMBER OF GRAINS	WEIGHT OF CALLUS AT COMMENCEMENT OF REGENERATION (g)	WEIGHT OF CALLUS AT TIME OF TEST (g)	NUMBER OF SHOOTS	NUMBER OF SHOOTS/GRAIN	NUMBER OF SHOOTS/WEIGHT OF CALLUS AT TIME OF TEST (g)
A: KOSHIHIKARI	63	7.62	56.61	50	0.79	0.88
B:KASALATH	73	6.87	70.39	451	6.18	6.41
C	87	15.65	145.49	1006	11.56	6.91
D	67	14.56	127.4	364	5.43	2.86

Fig. 2 Regeneration test of A: Koshihikari, B: Kasalath, and C: lines with improved regeneration selected from the BC4F2 population, cultivated by backcrossing and then self-fertilizing F1 individuals crossed from A and B, with A as the recurrent parent.

Mature seeds were cultured for one month in calli-inducing medium and the calli produced were transferred to regeneration medium where culture was continued for one month.

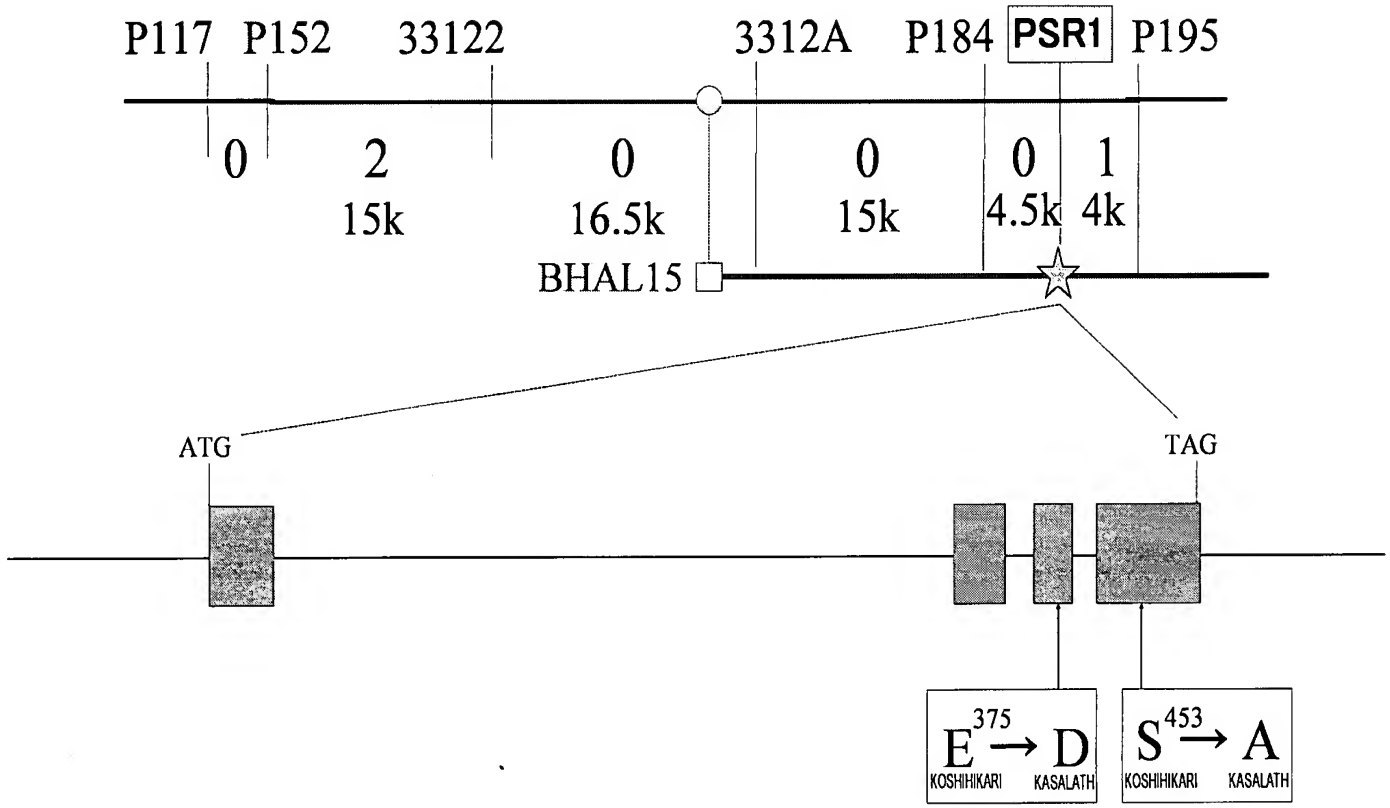


Fig. 3 High density linkage map and physical map of PSR1 gene.

As a result of linkage analysis, the PSR1 gene was located on the short arm of chromosome 1. Ferredoxin nitrite reductase was predicted to be within the 55kb candidate region, and analysis of the Koshihikari and Kasalath nucleotide sequences revealed nucleotide substitutions accompanied by amino acid mutations at two places in the exon.

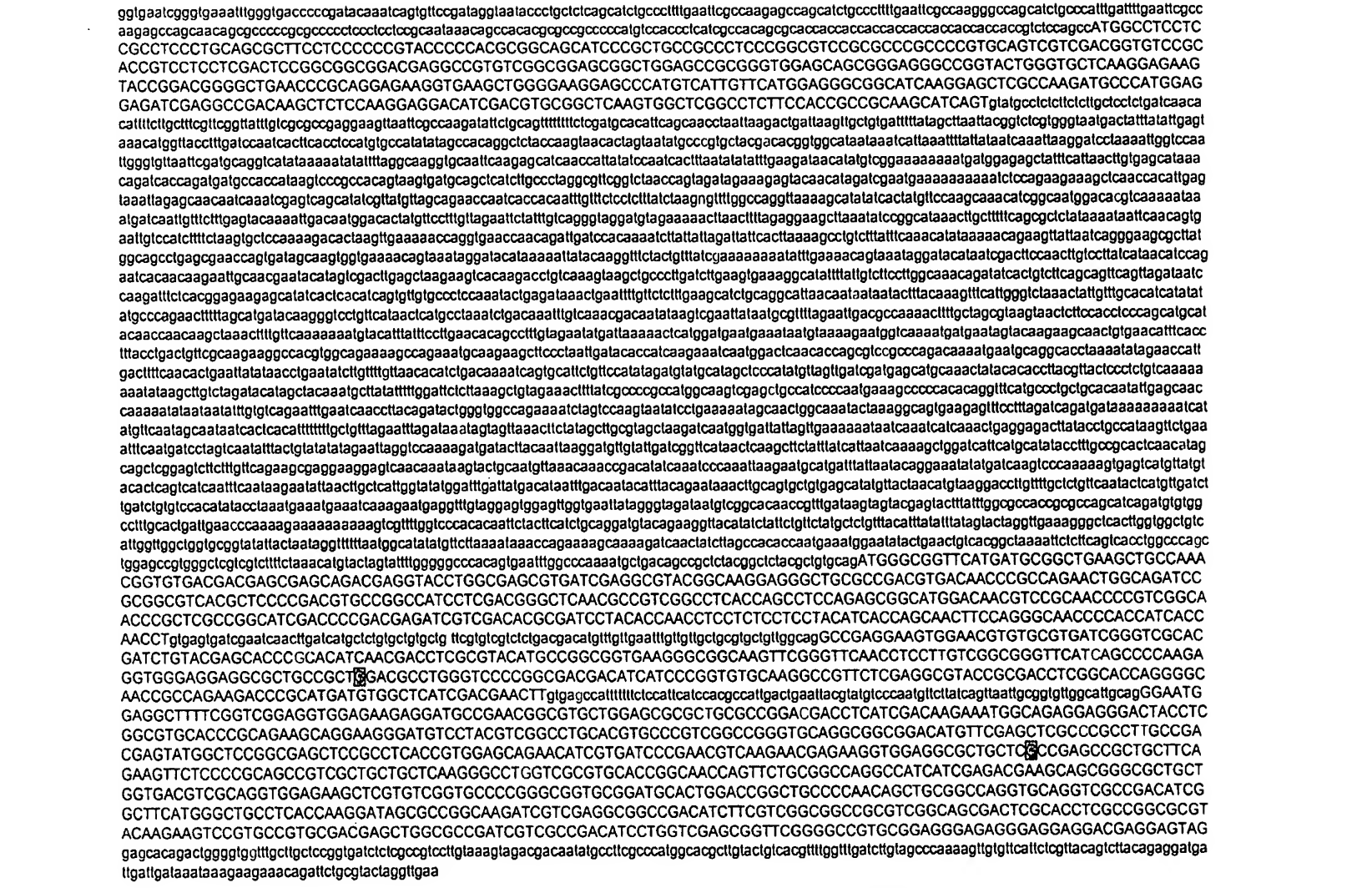


Fig. 4 Genomic nucleotide sequence of the Kasalath PSR1 gene

Lower case shows non-coding regions and introns. Upper case shows exons. Coloured letters show areas of difference when compared with Koshihikari. Green boxes: nucleotide substitutions accompanied by amino acid mutations; green letters: nucleotide substitutions not accompanied by amino acid mutations; blue letters: insertions; red letters: deletions.